SYMPOSIUM ON METABOLISM OF INORGANIC COMPOUNDS[1]

II. ENZYMATIC PATHWAYS OF NITRATE, NITRITE, AND HYDROXYLAMINE METABOLISMS[2]

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I. INTRODUCTION

The last decade has witnessed important advances in our knowledge of the enzymatic pathways and mechanisms of inorganic nitrogen metabolism as carried on by microorganisms, higher plants, and to a lesser extent, animals. Most of this new information has been concerned with the reduction of nitrate to nitrite, and more recently with the stepwise reduction of nitrite ultimately to the most highly reduced state of nitrogen as represented by ammonia and the amino group. In addition, it is only within the

last 2 or 3 years that segments of the biological process of nitrification, namely, the successive oxidation of ammonia to nitrite by *Nitrosomonas* and nitrite to nitrate by *Nitrobacter* have been attained for the first time at the cell-free level. Finally, the heretofore seemingly impregnable problem of preparing cell-free extracts capable of carrying out nitrogen fixation has at last been pierced, largely through the efforts of Mortenson, Mower, and Carnahan (79) as described in the next article of this Symposium.

The biological significance of inorganic nitrogen metabolism is evident from the realization that the ultimate source of nitrogen for all forms of life is inorganic nitrogen. From one point of view the evolution of biological systems has resulted in a particular nutritional relationship among organisms with respect to nitrogen utilization. We can regard nearly all plants and many microorganisms which are capable of converting the nitrogen atom from its various inorganic oxidized states (other than molecular nitrogen in most cases) to the more reduced forms, namely, ammonia and amino groups, as representing

the base or foundation of a typical ecological pyramid. Dependent upon this base are all the other forms of life (such as particular microorganisms and virtually all animals including man) who can fulfill their nitrogen requirement only from an exogenous supply of organic nitrogen and ammonia since they are incapable of transforming the more oxidized states of inorganic nitrogen to this level. Therefore, green plants and numerous microorganisms, by virtue of possessing the necessary complement of enzymes for carrying out nitrate assimilation, are ultimately responsible for providing nitrogen to many heterotrophic forms of life on our planet.

The essential features of inorganic nitrogen metabolism center about oxidation-reduction reactions. The nitrogen atom occurs in nature in a variety of oxidation states ranging from the disputed oxidation level of $+6$ as represented by the presumed short half-lived NO_3 (Wells (154)) through the oxidation states of $+5$ (N_2O_5 or its hydrated form, HNO_3); $+4$ (NO_2); $+3$ (N_2O_3 or its hydrated form, HNO_2); $+2$ (NO); $+1$ (N_2O), (HNO), H_2N_2O, and $NO_2 \cdot NH_2$ representing nitrous oxide, nitroxyl, hyponitrous acid (the dimer of the hypothetical nitroxyl), and nitramide, respectively); 0 (N_2); -1 (NH_2OH); -2 (NH_2NH_2); and -3 (NH_3). With the exception of the controversial $+6$ oxidation state, each has been implicated in the inorganic nitrogen metabolic pathways of either intact organisms or cell-free preparations.

The present review deals primarily with the enzymatic properties and sequences of nitrate, nitrite, and hydroxylamine transformation. In particular, emphasis will be placed on those papers published during the last 3 years which have a direct bearing on the over-all pathways and on the mechanisms of action of the steps concerned. The important contributions made in this area up until 1958 have been comprehensively and collectively treated in the McCollum-Pratt *Symposium on Inorganic Nitrogen Metabolism* (71), the symposium of the Society for Experimental Biology on "The Utilization of Nitrogen and Its Compounds by Plants" (111), and the review article by Nason and Takahashi (86). Portions of the earlier material have been included in the present paper, however, wherever it was deemed necessary for a more integrated presentation of the subject. Although it is evident that certain fundamental patterns are emerging

from the great welter of detail concerning the pathways of inorganic nitrogen metabolism, a number of important aspects are still not clear.

II. REDUCTION OF NITRATE TO NITRITE

At first glance there appears to be a variety of types of nitrate reduction in a wide range of microorganisms. Further examination has revealed, however, that they fall tentatively into two general classes: (i) *nitrate assimilation* or *assimilatory nitrate reduction*, and (ii) *nitrate respiration* or *dissimilatory nitrate reduction*. The two types of nitrate reduction play important roles in metabolic function and have been correlated in the past with certain underlying enzymatic constituents that are characteristically associated with each of the two processes. Further studies are revealing, however, that the enzymatic similarities between these two types are greater than their differences.

Nitrate assimilation represents the biological reduction of nitrate to ammonia or the amino level with the products being used for the biosynthesis of nitrogen-containing cell constituents, for example, proteins and nucleic acids. The transformation of nitrate to nitrite in the course of nitrate assimilation is the initial step in the enzymatic pathway of the 8-electron change required to attain the oxidation level of nitrogen (-3) as represented by the nitrogen of ammonia, amino acids, and proteins. In nitrate respiration, nitrate is used as the terminal electron acceptor in place of oxygen by several microorganisms under anaerobic or partially anaerobic conditions. The reduction products, which may include nitrite, nitric oxide (NO), nitrous oxide, molecular and other oxidation states of nitrogen, depending upon the organism and the chemical and physical conditions of its environment, are apparently not further utilized and are for the most part excreted into the surrounding medium. If molecular nitrogen, nitrous oxide, or nitric oxide is the product of nitrate respiration, the process is called *denitrification*. The latter is therefore a particular aspect of nitrate respiration which historically was designated by the term denitrification long before it was recognized to be essentially a form of nitrate respiration.

Because of its obvious physiological and enzymological similarity to oxygen respiration, it would be expected that nitrate respiration involves energy-yielding reactions which under

given conditions are necessary for the growth and well-being of the organism. At best there is slight evidence for the coupling of phosphorylation to nitrate respiration (Takahashi, Taniguchi, and Egami (136); Ohnishi and Mori (99)). It has not yet been demonstrated in cell-free preparations. Perhaps one of the most distinguishing enzymological features of nitrate respiration is the involvement of one or more cytochromes as electron carriers in the process. In contrast, the assimilatory reduction of nitrate to nitrite does not include any of the heme proteins as components of the electron transport system. Molybdenum, however, is a component of both systems.

A. Assimilatory Nitrate Reductase

1. The molybdoflavoprotein system. The first step in nitrate assimilation is catalyzed by the soluble, sulfhydryl molybdo-FAD-protein,[3] nitrate reductase. The enzyme was first characterized from *Neurospora* (Nason and Evans (85)) and soybean leaves (Evans and Nason (30)). *Neurospora* nitrate reductase is relatively specific for TPNH as the electron donor, whereas TPNH and DPNH are equally effective with the soybean enzyme. The presence of a similar or closely related nitrate reductase has since been reported to occur in a variety of higher plants (14, 37, 39, 98, 100, 117, 130, 142, 143) and indicated to be adaptive in nature (14, 37, 39, 117, 142, 143) as shown for *Neurospora* and other microorganisms (*see* review by Nason and Takahashi (86)).
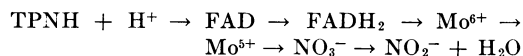
That molybdenum is the metal constituent of the *Neurospora* enzyme (Nicholas and Nason (90)) was shown by the (i) increased nitrate reductase activity in various protein fractions accompanied by increased molybdenum concentration, (ii) decrease in molybdenum concomitant with a decrease in enzyme activity during dialysis against cyanide, (iii) specific reactivation of the cyanide-dialyzed enzyme by molybdenum, and (iv) specific effect of molybdenum deficiency during growth resulting in decreased nitrate reductase activity.

[3] The following abbreviations are used: FAD, FADH₂, flavin adenine dinucleotide and reduced form; FMN, FMNH₂, flavin mononucleotide and reduced form; DPN, DPNH, diphosphopyridine nucleotide and reduced form; TPN, TPNH, triphosphopyridine nucleotide and reduced form; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

Nicholas and Nason (91) also demonstrated that flavin and molybdenum function as electron carriers in *Neurospora* nitrate reductase in the following sequence:

$$\text{TPNH} \rightarrow \text{FAD} \rightarrow \text{Mo} \rightarrow \text{NO}_3^-$$
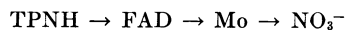
Flavin precedes molybdenum as shown by the inability of the molybdenum-free enzyme to catalyze the reduction of nitrate to nitrite by TPNH or reduced flavin, although FAD (or FMN) was reduced by TPNH. Added molybdenum specifically restored the ability of the enzyme to catalyze nitrite formation by reduced flavin, or TPNH plus flavin. That molybdenum functioned as an electron carrier was strongly implied by experiments in which reduced molybdate (prepared by treatment with sodium hydrosulfite) enzymatically reduced nitrate to nitrite, and by the observation that molybdate enzymatically oxidized FMNH_2. The reported isolation and separation of three oxidation states of molybdenum (Mo^{+6}, Mo^{+5} and Mo^{+3}) by Nicholas and Stevens (95) and their claim that Mo^{+5} was as effective as TPNH as an electron donor for the enzymatic reduction of nitrate support the suggestion by Nicholas and Nason (91) that molybdenum enzymatically undergoes a reversible oxidation-reduction from the +5 to the +6 oxidation states. Our present knowledge of the mechanism of action of nitrate reductase from *Neurospora* can be summarized as follows:

$$\text{TPNH} + \text{H}^+ \rightarrow \text{FAD} \rightarrow \text{FADH}_2 \rightarrow \text{Mo}^{6+} \rightarrow$$
$$\text{Mo}^{5+} \rightarrow \text{NO}_3^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$$

The observation by McElroy (70), Kinsky and McElroy (57), and Nicholas and Scawin (94) that inorganic phosphate stimulated the molybdenum-requiring step of the *Neurospora* nitrate reductase (namely, $\text{FADH}_2 \rightarrow \text{Mo} \rightarrow \text{NO}_3^-$) led Kinsky and McElroy (57) to postulate the existence of a phosphomolybdate complex in the enzyme. This is analogous to the situation in which specific reagents are known to reduce phosphomolybdenum complexes but not free molybdate. Final proof for the oxidation-reduction role of molybdenum in nitrate reductase, however, must await direct evidence from experiments with the endogenous molybdenum of the intact enzyme.

Studies have also been made with the nitrate reductase from soybean leaves identifying molybdenum (29, 92) as the metal component and

demonstrating the sequence and mechanism (92) to be essentially the same as in *Neurospora*. The presence of a somewhat similar system in *Escherichia coli*, in addition to the usual respiratory nitrate reductase, with FAD and molybdenum as components, has been indicated (93), depending upon the conditions of growth of the organism.

The findings of Kinsky and McElroy (57), that nitrate reductase preparations from *Neurospora* contained an inseparable TPN-cytochrome *c* reductase with somewhat similar properties, suggested that the two activities are catalyzed by the same enzyme. Adaptation experiments showed that both nitrate reductase and cytochrome *c* reductase activities were induced in a parallel manner when *Neurospora* was grown in a medium containing varying amounts of nitrate. However, there was no nitrate reductase in mycelia grown on ammonia as the sole nitrogen source, although there was substantial TPN-cytochrome *c* reductase having approximately 50% of the specific activity of that obtained from nitrate-grown *Neurospora*. Their results point to two kinds of TPN-cytochrome *c* reductases: (i) a constitutive enzyme with no as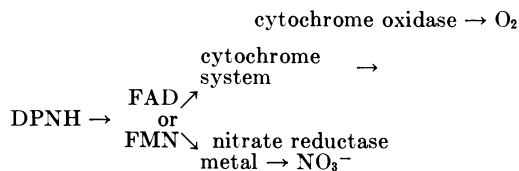sociated nitrate reductase as indicated by the adaptation experiments with ammonia-grown mycelia, and (ii) an enzyme associated with nitrate reductase activity. The latter enzyme may well be a single system which is responsible for nitrate reduction, and in part for cytochrome *c* reduction; or the two activities share a common enzymatic step, presumably the rate-limiting TPN-flavin reductase reaction. Since several metal-binding agents were potent inhibitors of nitrate reductase but not of cytochrome *c* reductase, it seems plausible that the next step would involve the enzymatic transfer of electrons from flavin, either to nitrate via molybdenum, thus giving nitrate reductase activity, or to cytochrome *c* to give cytochrome *c* reductase activity. The parallel inducement of both activities in nitrate-grown mycelia could be ascribed primarily to the adaptive formation of nitrate reductase, the first step of which (TPNH → flavin) could also be used by the cytochrome *c* reductase, thus reflecting itself in an increased cytochrome *c* reductase activity. This is represented as follows:

$$\text{TPNH} \rightarrow \text{FAD} \begin{array}{c} \nearrow \text{Mo} \rightarrow NO_3^- \\ \text{metal-binding agents} \\ \searrow \text{cytochrome } c \end{array}$$
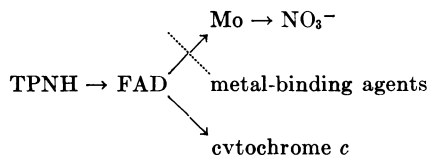
The above hypothesis implies that at least two component enzymes are involved in the nitrate reductase system, although they have not as yet been separated.

Silver (127) observed a pyridine nucleotide-nitrate reductase in the extracts of the yeast *Hansenula anomala* grown on a nitrate-containing medium. The enzyme was similar to that found in *Neurospora* and soybean leaves, having a metalloflavoprotein with FAD and molybdenum as probable prosthetic groups. Spectrophotometric observations eliminated a direct role of the cytochromes in nitrate reduction. Taniguchi and Ohmachi (140) have recently studied an inducible pyridine-nucleotide nitrate reductase residing in the large particles of nitrate-grown *Azotobacter* cells. Except for its particulate nature, the enzyme strongly resembles that observed in *Neurospora* and higher plants, and is apparently of the assimilatory type. It is a sulfhydryl, metalloenzyme in which no cytochromes participate. Added FAD or FMN is significantly stimulatory with DPNH giving twice as much maximal activity as TPNH. The same large particles which contain nitrate reductase also possess DPNH oxidase activity. The latter activity in contrast to that of nitrate reductase involved the participation of cytochrome components. The following proposed sequence (140) of electron transfer in *Azotobacter* from DPNH bears a strong similarity to the suggested scheme indicated above for *Neurospora*:

$$\text{DPNH} \rightarrow \begin{array}{c} \text{FAD} \nearrow \\ \text{or} \\ \text{FMN} \searrow \end{array} \begin{array}{c} \text{cytochrome} \\ \text{system} \end{array} \rightarrow \begin{array}{c} \text{cytochrome oxidase} \rightarrow O_2 \\ \\ \text{nitrate reductase} \\ \text{metal} \rightarrow NO_3^- \end{array}$$

If the above unidentified metal should prove to be molybdenum, then the nitrate reductase of *Azotobacter* would be essentially the same as that already described for *Neurospora* and higher plants.
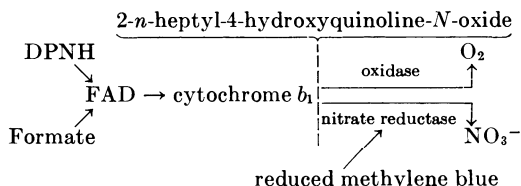
2. Photoreduction of nitrate. Although the effect of illumination in accelerating the reduction of

nitrate in green plants has been known for some time, the explanation of this phenomenon has been a highly controversial one (*see* reviews by Nightingale (96, 97), Street (135), and Virtanen and Rautanen (148)). That photochemical processes may furnish the hydrogen donors (i.e., reduced pyridine nucleotides) for nitrate reduction was shown by Evans and Nason (30) through the use of grana or broken chloroplasts and purified nitrate reductase from soybean leaves. A similar concept based upon a competition between nitrate and carbon dioxide for reducing power has been proposed by Van Niel, Allen, and Wright (146) as a result of their experiments with *Chlorella*. They observed that at low light intensity the uptake of carbon dioxide, but not the production of oxygen, was decreased in the presence of nitrate. Kessler (55), in discussing the enhanced reduction of nitrate by light, concluded from various lines of evidence that although the photochemical reduction of pyridine nucleotides is likely to be one of the important factors, it cannot explain all the available experimental results. He feels that the action of light is a complicated phenomenon contributing to the supply of hydrogen donors, energy-rich phosphate bonds, and carbon compounds. In this respect it is of interest that Stoy (133), in testing the possible role of riboflavin as a light-absorbing catalyst in biological photoreduction, showed that photochemically reduced riboflavin is a more efficient electron donor than DPNH with the use of purified nitrate reductase of wheat leaves. He had previously observed (132) a significant increase in nitrate reduction by detached wheat leaves in the blue and violet parts of the spectrum, suggesting involvement of a yellow pigment.

B. Respiratory Nitrate Reductase

1. Studies of the enzyme system from E. coli. The first demonstration of nitrate respiration in nondenitrifying bacteria was made by Quastel, Stephenson, and Wetham (114) in 1925. They observed that their strain of *E. coli* failed to grow anaerobically on lactate unless a suitable electron acceptor such as nitrate was furnished in the medium. Subsequent experiments by Yamagata (156), with cell-free preparations from aerobically grown *E. coli* and reduced methylene blue as the electron donor for the reduction of nitrate, pro-

vided direct proof for the existence of the enzyme nitrate reductase. Detailed investigations some years later by Egami and his collaborators indicated that the nitrate-reducing system of *E. coli* was bound to a subcellular particle which was also intimately involved in aerobic respiration. On the basis of their experiments they formulated (141) in 1956 the following sequence of electron transport involving the reduction of nitrate and oxygen:

$$
\begin{array}{c}
\text{2-}n\text{-heptyl-4-hydroxyquinoline-}N\text{-oxide} \\
\text{DPNH} \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \text{O}_2 \\
\searrow \quad\quad\quad\quad \text{oxidase} \quad\quad \uparrow \\
\text{FAD} \rightarrow \text{cytochrome } b_1 \\
\nearrow \quad\quad\quad\quad \text{nitrate reductase} \downarrow \\
\text{Formate} \quad\quad\quad\quad\quad\quad\quad\quad \text{NO}_3{}^- \\
\nearrow \\
\text{reduced methylene blue}
\end{array}
$$

In support of this scheme were the observations that the rate of oxidation of DPNH by nitrate was considerably increased by catalytic quantities of FAD. Cytochrome b_1 was implicated by (i) spectral studies showing oxidation of the reduced heme complex upon addition of nitrate in the absence of oxygen, and (ii) the large inhibition caused by 2-n-heptyl-4-hydroxyquinoline-N-oxide. The latter is considered to be a specific inhibitor of cytochromes b and b_1 (66). In this system, nitrate reductase is defined as the terminal member of the electron transport chain reacting directly with the nitrate. It was concluded that nitrate served as a cellular oxidant through the action of the above terminal nitrate reductase during anaerobiosis, in place of oxygen and the terminal respiratory oxidase. Except for the terminal steps, the same electron transport chain appeared to be involved in both the aerobic and anaerobic states. As seen in the above sequence, the use of reduced methylene blue or other suitable reduced dye (e.g., reduced methyl viologen) offers a distinct advantage for the study of the terminal nitrate reductase itself apart from the other members of the organized electron transport chain.

Iida and Taniguchi (41), in further studies of the complex *E. coli* particulate electron transport system extending from DPNH or formate to nitrate, confirmed the sequence proposed above. They found in addition that an unidentified acid-labile, heat-stable, soluble factor (not replaceable by FAD, menadione, or ferrous ion, singly or in combination) was necessary for full

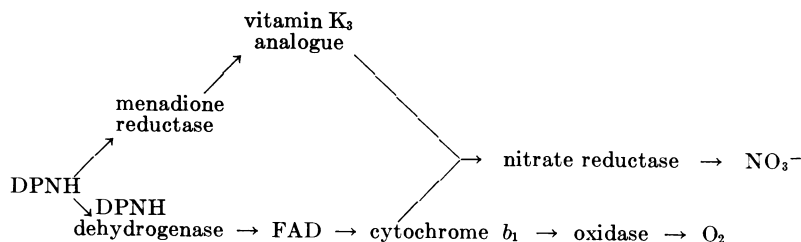activity of the DPNH- or formate-nitrite reductase system and the DPNH oxidase. Menadione was observed to reverse partially the inhibition caused by Dicumarol. Solubilization by means of isobutanol and deoxycholate treatment of the organized electron transport system resulted in its inactivation. The marked inhibition of the terminal nitrate reductase (using reduced methylene blue as the electron donor) by several metal-binding agents suggested the presence of a metal component.

When the same *E. coli* strain was grown aerobically in a medium similar to that used by Nicholas and Nason (93) for *E. coli* strain B, with the exception that yeast extract was also added, it was observed by Itagaki and Taniguchi (48) that a considerably increased amount of soluble nitrate reductase could be easily extracted from the cells. Although the soluble crude extract in contrast to the particulate fraction was incapable of using formate as an electron donor, it possessed DPNH-nitrate reductase activity (TPNH was less effective) which was also sensitive to 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide. It apparently contained a small quantity of actively participating cytochrome b_1 ; and, after dialysis, was markedly stimulated by the addition of FAD, menadione, and Fe^{++} ions. The latter could also serve as an electron donor; and Dicumarol inhibition was shown to be reversed by menadione. On the basis of their results, Itagaki and Taniguchi (48) proposed that the soluble DPNH-nitrate reductase system of the *E. coli* Yanagutchi strain, grown as described above, consists of the following sequence:

$$DPNH \rightarrow \begin{matrix} FAD \\ or, menadione, \\ FMN \end{matrix} \quad \begin{matrix} Fe^{++} \\ \Updownarrow \\ Fe^{+++} \end{matrix} \quad \rightarrow$$

$$cytochrome\ b_1 \xrightarrow[\text{(unknown heavy metal)}]{\text{nitrate reductase}} NO_3^-$$
quinoline oxide
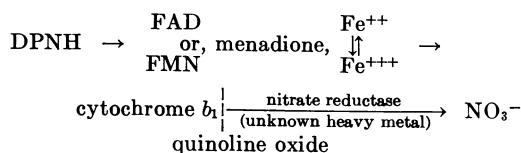
The marked inhibition of the terminal nitrate reductase by cyanide and azide implied the presence of a heavy metal component as indicated above. The proposed scheme is essentially in agreement with the results obtained some 5 years earlier by Wainright (149), who observed that the pyridine nucleotide-nitrate reductase, in a cell-free preparation of aerobically grown *E. coli* strain 1433, probably included a cytochrome component and was stimulated by menadione and ferrous ions in the presence of added FAD or FMN. Studies by other workers with intact cells and extracts of *E. coli* had also implicated the participation of cytochrome (9) and iron (28) in the nitrate reduction process.

Heredia and Medina (38, 75), with extracts of aerobically grown *E. coli* strain 86, obtained evidence for two nitrate reductase pathways of a particulate nature. They concluded that through the action of menadione reductase (155), vitamin K_3 (menadione) or one of its analogues serves as an electron carrier in one of the pathways for the ultimate enzymatic reduction of nitrate by DPNH under aerobic as well as anaerobic conditions. The strikingly inhibitory effects of several metal-binding agents indicated the involvement of a metal constituent. The addition of menadione to their enzyme system proved to be absolutely essential for the reduction of nitrate aerobically and caused as much as a 30-fold stimulation in the reduction of nitrate anaerobically by the same preparations. The fact, however, that in the absence of added menadione there was still some anaerobic nitrate reduction which was stimulated by FAD, suggested that a second mechanism of nitrate reduction is also present, probably of the oxygen-sensitive respiratory type already indicated by Egami and his collaborators (141). They proposed the following relationship between the two pathways:

$$
\begin{array}{c}
\text{vitamin } K_3 \\
\text{analogue} \\
\nearrow \qquad \searrow \\
\text{menadione} \\
\text{reductase} \\
\nearrow \qquad \qquad \qquad \searrow \\
DPNH \qquad \qquad \qquad \nearrow \rightarrow \text{nitrate reductase} \rightarrow NO_3^- \\
\searrow DPNH \qquad \qquad \nearrow \\
\text{dehydrogenase} \rightarrow FAD \rightarrow \text{cytochrome } b_1 \rightarrow \text{oxidase} \rightarrow O_2
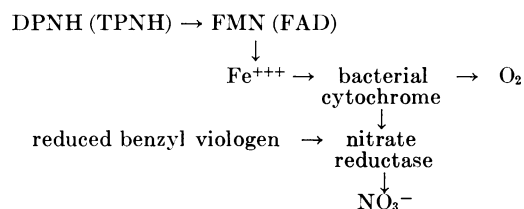\end{array}
$$

The postulated scheme of Medina and Heredia for the involvement of menadione in the enzymatic reduction of nitrate by *E. coli* differs from that previously indicated by Itagaki and Taniguchi (48). Further experimentation is necessary to resolve the differences between the electron transport sequences proposed by these two groups.

Using *E. coli* grown anaerobically in a medium containing 0.1% KNO_3, Taniguchi and Itagaki (137, 138) obtained a particulate formate-nitrate reductase system essentially similar to that already indicated earlier for the aerobically grown cells (141) but possessing a 20- to 30-fold greater specific activity. In contrast to the particulate system of aerobically grown cells, the particles were devoid of DPNH and TPNH oxidases as well as various dehydrogenases with the notable exception of formic dehydrogenase. Solubilization of the enzyme by alkaline cold incubation after heat treatment (or by steapsin or chymotrypsin digestion) followed by purification to a homogenous state yielded only the terminal nitrate reductase (reduced methyl viologen as electron donor) of the original particulate system. The purified enzyme contained neither formic dehydrogenase, flavin, nor cytochrome b_1, and possessed a molecular weight of 1 million. The difference spectrum (oxidized minus reduced) of the homogeneous enzyme preparation displayed a broad peak at 445 to 450 mμ, which instantly disappeared under anaerobic conditions upon addition of nitrate, accompanied by the simultaneous production of nitrite. Spectrographic analysis showed the presence of 1 atom of bound molybdenum and about 40 molecules of bound iron per protein molecule (138). Iida and Yamasaki (42) at the same time also reported the presence of molybdenum and iron in the nitrite reductase of *E. coli*.

A comparison of the properties of the assimilatory nitrate reductase of *Neurospora* with those of the respiratory nitrate reductase of *E. coli* is given in Table 1. Both systems share at least one fundamental characteristic, namely, the possession of molybdenum as an active enzymatic component. For the *Neurospora* enzyme, the evidence indicates that molybdenum serves as an electron carrier (apparently undergoing a reversible change in oxidation state from +6 to +5) to nitrate. A similar mechanism of action of molybdenum has now been implicated for the *E. coli* enzyme (49). The recent finding of molybdenum

in the respiratory nitrate reductase of *E. coli* lends further support to the generally emerging pattern that molybdenum is a necessary component of those enzymes capable of catalyzing the reduction of nitrate to nitrite (the nitrate reductase of *Neurospora* (90), higher plants (92), and *E. coli* (42, 138); xanthine oxidase from milk and liver (26, 116); and aldehyde oxidase (68)).

2. Studies of the enzyme system from other microorganisms. Sadana and McElroy (123) purified and characterized a nitrate-reducing system from the salt-water luminous bacterium, *Achromobacter fischeri*, and proposed the following pathway of electron transport:

DPNH (TPNH) → FMN (FAD)
$$\downarrow$$
$$Fe^{+++} \rightarrow \text{bacterial} \rightarrow O_2$$
$$\text{cytochrome}$$
$$\downarrow$$
reduced benzyl viologen → nitrate
reductase
$$\downarrow$$
$$N\overset{\downarrow}{O}_3{}^-$$

The electron transport chain was separated into two soluble portions, (i) the electron-donor system, namely, DPNH-cytochrome *c* reductase with a requirement for FMN or FAD, and (ii) the terminal nitrate reductase system, by which electrons were transported from the reduced cytochrome to nitrate. The cytochrome component which was auto-oxidizable had absorption bands similar to mammalian cytochrome *c*. When reduced benzyl viologen supplied electrons, the bacterial cytochrome was not involved as indicated by spectral studies as well as the lack of inhibition by CO in contrast to the photoreversible inhibition by this reagent with DPNH as the electron donor. The proposed electron transport chain is similar in some respects to that already indicated for *E. coli*. In view of the cytochrome involvement and the apparent competition by O_2 for electrons in the conversion of nitrate to nitrite, the nitrate-reducing system of *A. fischeri* is classified under nitrate respiration.

A somewhat similar system has been reported by Fewson and Nicholas (32) in the denitrifying bacterium *Pseudomonas aeruginosa*. The purified DPNH-specific, sulhydryl flavoenzyme contained cytochrome *c* and apparently involved molybdenum as a component of the system. The following sequence of electron transport was suggested:

TABLE 1. *Comparison of Neurospora nitrate reductase (nitrate assimilation type) with Escherichia coli nitrate reductase (nitrate respiration type)*

Properties	*Neurospora* nitrate reductase	*E. coli* nitrate reductase
1. Original physical state	Soluble, molecular weight not reported	Particulate, mol wt ca. 1,000,000
2. Sensitivity to O_2	Unaffected	Decreases enzyme activity by competing for electrons
3. Electron donors	TPNH, $FADH_2$, $FMNH_2$, Mo^{5+}, reduced indophenol dyes, other artificial dyes not reported	DPNH, $FADH_2$, reduced cytochrome b_1, various reduced dyes (including reduced methylene blue, methyl viologen, benzyl viologen, phenosafranine, etc.)
4. Components of electron transport chain		
Flavin	FAD	FAD
Cytochromes	None	Cytochrome b_1
Mo	Present	1 atom/enzyme molecule
Fe	Not reported	Ca. 40 atoms/enzyme molecule possibly
Vitamin K derivative	Not reported	Possibly
Other factors	Not reported	Unidentified soluble factor
5. Sequence of electron transport chain	TPNH $\rightarrow$ FAD $\rightarrow$ Mo $\rightarrow$ NO_3^-	DPNH $\rightarrow$ FAD, menadione(?), $\begin{matrix}Fe^{++}\\ \Updownarrow \\ Fe^{+++}\end{matrix}$ (?) cytochrome $b_1 \rightarrow$ Mo $\rightarrow$ NO_3^- $\begin{matrix}Fe^{++}\\ \Updownarrow \\ Fe^{+++}\end{matrix}$ (?)
6. Separation of nitrate reductase activity from remainder of electron transport chain	Not reported	Cytochrome $b_1 \rightarrow$ Mo $\rightarrow$ NO_3^- $\begin{matrix}Fe^{++}\\ \Updownarrow \\ Fe^{+++}\end{matrix}$ (?)
7. Other activities associated with electron transport chain	TPNH-cytochrome c reductase activity	a) With intact chain: formate dehydrogenase, and in some preparations other dehydrogenases and reduced pyridine nucleotide oxidases b) Separated nitrate reductase: none of the above activities other than nitrate reductase
8. K_m for nitrate	1.4×10^{-3} M	5.1×10^{-4} M
9. Metabolic role	Nitrate assimilation	Nitrate respiration

$$\text{DPNH} \rightarrow \text{FAD} \rightarrow \text{cytochrome } c \rightarrow \text{Mo} \rightarrow NO_3^-$$
$$\searrow \text{cytochrome oxidase}$$
$$\searrow O_2$$

In extending their earlier reports (16, 17) of a particulate DPNH-specific nitrate reductase from the *Rhizobium japonicum* of soybean nodules, Cheniae and Evans (18) concluded that the system compared most favorably with the nitrate-respiratory type from *E. coli*. The enzyme was a sulfhydryl protein which was inhibited by various metal-binding agents, antimycin A, and Dicumarol. It exhibited a menadione requirement and possibly involved the cytochrome b complex. Thus far the participation of flavin has not been shown. Succinate or several reduced dyes served as an electron source in place of DPNH. Cheniae and Evans (19) are still of the opinion that the nodule nitrate reductase system is related in some manner to the nitrogen fixation process. They found that the activity of the enzyme in *Rhizobium* from nodules of soybean

plants grown without combined nitrogen was at least 8- to 10-fold greater than that of pure cultures of *Rhizobium* grown in media containing nitrate.

A unique nitrate reductase, apparently of the respiratory type, from *Aerobacter aerogenes* was investigated by Pichinoty and Senez (104, 108). The enzyme which apparently catalyzed the first step in the ultimate conversion of nitrate to ammonia neither utilized pyridine nucleotides nor contained cytochromes. Molecular hydrogen which served as the electron source for nitrate reduction to nitrite (as well as to ammonia) stoichiometrically reduced nitrate in the presence of benzyl viologen:

$$H_2 \xrightarrow[\text{hydrogenase}]{} \text{benzyl viologen} \xrightarrow[\substack{\text{nitrate}\\\text{reductase}}]{} NO_3^-$$
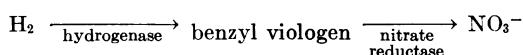
The system was inhibited by a number of metal-binding agents.

C. Factors Influencing the Pathways of Nitrate Reduction

Walker and Nicholas (153) recently reported that *Neurospora* grown at low oxygen tensions exhibited the enzymatic and other metabolic properties characteristic of nitrate respiration, in contrast to that of nitrate assimilation when grown under a more aerobic environment. Under the former conditions nitrate reductase was dependent on iron as well as molybdenum in the nutrient medium; nitrite accumulated and cytochromes b and c increased in the mycelia. When oxygen was no longer limiting, the nitrite which had accumulated earlier was now reutilized and nitrate reductase activity became independent of the iron status of the medium.

The data of Walker and Nicholas have essentially confirmed the earlier report of Lenhoff, Nicholas, and Kaplan (65) and that of Higashi (40), who indicated that the oxygen tension of the medium during the growth of *Pseudomonas fluorescens* determined the alternative routes of terminal electron transfer formed by the organism. Lenhoff et al. had observed that cells grown at low oxygen tension in a nitrate-containing medium had large quantities of cytochrome pigment, a higher iron requirement, and a lower molybdenum requirement than those grown under more aerobic conditions. Their data suggested that in aerobically grown cells the molybdoflavoprotein functioned primarily in the nitrate assimilatory process, whereas in anaerobically

grown cells the cytochrome-nitrate reductase chain served in terminal respiration (5).

Earlier findings by Sacks (121), Allen and Van Niel (5), Baalsrud and Baalsrud (10), and Rosenberger and Kogut (118) had already attributed the typically pink color of denitrifying bacteria, when grown under anaerobic conditions in the presence of nitrate, to an increase in bacterial cytochrome. These results, in addition to providing further support for our current concept regarding the physiological roles of nitrate respiration and assimilation, also illustrated the adaptability of the two processes within the same organism, with one pathway often dominating the other, depending, among other factors, upon the availability of molecular oxygen.

D. Adaptation of Nitrate Reductase

The literature dealing with the adaptive nature of nitrate-reducing enzyme systems has been collectively reviewed through 1957 by Kluyver (59), Delwiche (24), and Nason and Takahashi (86). Early studies, particularly by Pollock (109), with nitrate-respiring organisms including the denitrifiers demonstrated the adaptive nature of the nitrate-reducing enzyme system. This was indicated by a lag period in the reduction of nitrate by cells not originating in a nitrate-containing medium. Wainwright and Nevill (150), using nitrogen-depleted *E. coli*, observed a marked increase in nitrate reductase activity with a maximal value being attained within 2 hours after adding nitrate. The striking stimulation brought about by the addition of casein hydrolyzate suggested that the limiting factor in adaptation was the slow rate of synthesis de novo of amino acids. They also showed that auxotrophic mutants of *E. coli* requiring specific amino acids did not grow or form nitrate reductase in the absence of the appropriate amino acid. Their results with mutants requiring uracil or thymine suggested that ribonucleic acid synthesis is necessary for the synthesis of the nitrate-reducing system. Egami, Hayase, and Taniguchi (27) were unable to induce the formation of formic dehydrogenase and nitrate reductase (cytochrome $b \rightarrow NO_3^-$) in an *E. coli* mutant auxotrophic for hemin in aerobic or anaerobic cultures in the presence or absence of hemin.

Pichinoty and D'Ornano (105, 106) concluded that the adaptive formation of nitrate reductase activity in cell suspensions of *Aerobacter aerogenes* under anaerobic conditions is due to syn-

thesis de novo of the enzyme from free amino acids. The induced formation of nitrate reductase in this organism ($H_2 \rightarrow$ benzyl viologen $\rightarrow NO_3^-$) by nitrate or nitrite ions was markedly stimulated by the addition of casein hydrolyzate to the medium and was prevented by chloramphenicol. Molecular oxygen was reported to inhibit reversibly enzyme activity as well as adaptive formation of the enzyme. The latter phenomenon, namely that the adaptation of cells is reversibly inhibited by oxygen, had already been demonstrated by Pollock (110) for *E. coli*. The classic observation of little or no denitrification in denitrifying organisms subjected to aerobic conditions may well reside in the inhibition of both the biosynthesis and activity of the nitrate reductase.

Although most of the evidence illustrated the adaptive nature of the respiratory nitrate reductase in microorganisms, there are a few reports in the literature which fail to support this viewpoint. Straughn (134) was unable to obtain an enhanced nitrate reductase activity by the addition of nitrate to cells grown in a peptone broth. Farkas-Himsley and Artman (31) found that although the kinetics of nitrate reduction by nonproliferating suspensions of old *E. coli* cells with and without nitrate was suggestive of an adaptive system, it could be ascribed instead to a permeability barrier in old cells toward nitrate. Although old *E. coli* cells grown on a medium supplemented with nitrate reduced nitrate at a faster rate and without a lag period as compared to similar cells from a nitrateless medium, the cell-free extracts from both groups reduced nitrate at the same rapid rate. In further support of their viewpoint was the observation that nonproliferating young cells reduced nitrate at the same fast rate regardless of the presence or absence of nitrate in the growth medium. In general their results are suggestive of a substrate-inducible permease.

The adaptive nature of the assimilatory nitrate reductase (flavomolybdoprotein) appears to be clearly established in *Nuerospora* (85) and higher plants (14, 17, 37, 39, 142, 143). Most of the evidence is based on the demonstration that cell-free preparations of tissues exposed to ammonium salts or amino acids showed little or no nitrate reductase activity in comparison to the high enzymatic activity in nitrate-grown material. The markedly inhibiting effect of ammonia on the nitrate reductase of certain fungi (not including *Neurospora*) has been ascribed by Morton (80) to

its adverse effect on the formation and stability of the enzyme.

E. Nitrate Reductase Activity in Animal Tissues

The ability of animal tissues to reduce nitrate was first demonstrated in liver preparations of various species by Bernheim and Dixon (11). They showed that nitrate was enzymatically reduced by simply functioning as a hydrogen acceptor of aldehyde oxidase in place of methylene blue or oxygen. The recent studies of Omura and Takahashi (101, 102), however, have led to the suggestion that the nitrate reductase activity of animal cell preparations may not necessarily be due to the aldehyde oxidase and xanthine oxidase activities. Further evidence on this point and on the possible metabolic significance of nitrate reduction in animal tissues is necessary, however, before any conclusions can be drawn.

III. Stepwise Reduction of Nitrite

With the continued elucidation of the enzymatic mechanisms for the conversion of nitrate to nitrite, attention has been turning increasingly to the subsequent reduction of nitrite. Although there has been some degree of success in separating and isolating one or two of the enzymatic steps presumed to be involved in the ultimate reduction of nitrate via nitrite to ammonia, the pathway, intermediates, and mechanisms beyond the nitrite stage are still very much in need of clarification. Perhaps most indicative of our limited knowledge in this area is the fact that it has not yet been conclusively established whether the subsequent reduction of nitrite by microorganisms and higher plants proceeds via organic intermediates or by way of an inorganic pathway. The present status of the problem can best be evaluated by indicating the progress that has been made to date in various aspects of the field.

If we assume that the biological reduction of nitrite proceeds via the inorganic pathway and that two electron changes are involved for each enzymatic step, then the following sequence of intermediates with the indicated oxidation states for the nitrogen atom can be postulated.

$$NO_2^- \rightarrow (HNO), NO_2 \cdot NH_2,$$
$$+3 \qquad\qquad +1$$

$$or\ H_2N_2O_2 \rightarrow NH_2OH \rightarrow NH_3$$
$$-1 \qquad\quad -3$$
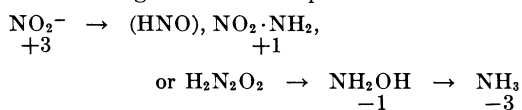
The enzyme concerned with the reduction of nitrite to the +1 oxidation state would be called

nitrite reductase, whereas that which mediates the reduction of hydroxylamine to ammonia is hydroxylamine reductase. We are still very uncertain as to the +1 oxidation state nitrogen intermediate in the inorganic pathway. As seen from the above sequence at least three possibilities exist: the hypothetical nitroxyl (HNO), nitramide ($NO_2 \cdot NH_2$), and hyponitrous acid ($H_2N_2O_2$). The evidence that hyponitrous acid (and nitramide) may or may not be involved in biological systems is discussed below.

A. Nitrite Reductase in Nondenitrifying Organisms

Yamagata (157) was the first to demonstrate the presence of nitrite reductase by using cell-free preparations of *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*). Subsequently Taniguchi et al. (139) observed the enzyme in *Bacillus pumilus* with the use of reduced methylene blue as the electron donor. The detection of pyridine-nucleotide-nitrite reductase in extracts of *Neurospora* and soybean leaves (30, 85) subsequently led to partial purification and characterization of nitrite and hydroxylamine reductases from these sources (84, 119, 159). Both enzymes in *Neurospora*, which catalyzed the reduction of their respective substrates to ammonia, were shown to be metalloflavoproteins with unidentified metal components, whereas the corresponding systems from soybean leaves appear at this time to be somewhat different, exhibiting a specific requirement for Mn^{++} and an as yet unidentified cofactor (119).

The nitrite and hydroxylamine enzymes have been recently purified from soybean leaves by Roussos and Nason (119) and shown to require substrate quantities of nitrite and hydroxylamine, respectively, in order to catalyze the oxidation of reduced pyridine nucleotides. Both enzyme activities are inhibited by several metal-binding agents and are stimulated 3- to 6-fold specifically by Mn^{++}. They also show an absolute requirement for an unidentified, dissociable, heat-stable organic factor obtained from soybean leaf extracts. DPN was demonstrated to be one of the products of both the nitrite and hydroxylamine enzyme reactions but the fate of nitrite and hydroxylamine is still unknown, although the latter disappeared in quantities stoichiometric with the oxidation of DPNH. The failure to demonstrate either that flavin was required or that ammonia was an end product of the enzy-

matic reactions suggested the possibility that a fragmentation of the nitrite reductase and hydroxylamine reductase chains had been achieved.

Recent studies of a 50-fold purified *Neurospora* nitrite reductase by Nicholas, Medina, and Jones (89) failed to yield substantially more conclusive information about its properties. No mention was made of the nitrite reduction product of the enzymatic reaction; whether it was ammonia or a substance of an intermediate oxidation state was not indicated. The tentative identification of the native flavin as FAD (no data were presented) and the conclusion based on the following evidence that iron and copper were also active constituents of the enzyme system must await further experimental verification. The nitrite reductase activity of extracts of mycelia deficient in Mg, Fe, Cu, or Zn was significantly depressed, whereas a deficiency in Mo or Mn was without effect on the enzyme. Although the addition of iron compounds (Fe^{+++}) and copper compounds (Cu^{++}) to extracts of Fe-deficient and Cu-deficient mycelia, respectively, markedly stimulated enzyme activity, there was no indication of the specificity of these metal effects. Nor were any attempts reported to remove the metal component from the purified enzyme by dialysis against metal-binding agents followed by restoration studies, a procedure which proved to be of great value in the identification of molybdenum as the metal constituent of nitrate reductase (90). The copper and iron contents of various enzyme fractions, however, were shown to be related to their nitrite reductase activities. Cuprous ion was implicated as an electron donor in the enzymatic reduction of nitrate, whereas ferrous ion and reduced cytochrome *c* were inactive in this respect. The above Mg requirement was attributed to its indirect effect in enzyme formation, since it did not accumulate in the purified fractions of the enzyme nor did its addition in vitro activate the nitrite reductase. The zinc requirement, however, was ignored. The observation that the spectra of purified enzyme fractions reduced with dithionite or DPNH showed no definite cytochrome bands contradicted an earlier report from the same laboratory (87) in which cytochromes *b* and c_1 were identified in the purified *Neurospora* nitrite reductase. The latter report also claimed that (i) the enzyme was particulate, (ii) hyponitrite was the product of the reaction, and (iii) there was a "phosphorylation during nitrite reductase action because uncoupling reagents inhibited the enzyme

and inorganic phosphate was incorporated into the ATP." These points were notably absent in the recent and more detailed presentation of the properties of the enzyme by the same group (89) and one can only assume that they no longer apply. With regard to the last point, Kessler and Bucker (56), on the basis of experiments with green algae, concluded that the reduction of nitrite *required* (rather than *generated*) high energy phosphate in contrast to the first step of nitrate reduction where it is not necessary.

The pyridine nucleotide-nitrite reductase of *Azotobacter* was shown by Spencer, Takahashi, and Nason (131) to be an adaptive FAD-metalloprotein which catalyzed the reduction of nitrite to ammonia. Unlike hydroxylamine reductase from the same organism, it exhibited no requirement for manganous ions. Lazzarini and Atkinson (64) characterized a cyanide-sensitive TPNH-specific nitrite reductase from *E. coli* which was not stimulated by FMN or FAD or a variety of metal ions. Although the reduction product of the reaction was ammonia, the enzyme was inactive toward nitrous oxide and hyponitrite (and nitrate), but rapidly reduced hydroxylamine. In fact, TPNH-specific hydroxylamine reductase activity closely paralleled the nitrite reductase activity throughout the 100- to 200-fold purification. All attempts to alter the proportions of the two activities were unsuccessful, indicating that the same enzyme probably accounted for both activities. Interestingly enough the evidence obtained with isotopically labeled nitrite indicated that *free* hydroxylamine was not an obligate intermediate in the reduction of nitrite to ammonia. It seems possible that one enzyme catalyzes the complete reduction of nitrite and hydroxylamine to ammonia without the intervention of free intermediates. The purified enzyme also had TPNH-specific cytochrome *c* and sulfite reductase activities. The latter activity is especially noteworthy in view of the recent results of Mager (67) implicating the TPNH-hydroxylamine reductase activity of *E. coli* as secondary to the TPNH-sulfite reductase which it always accompanied (*see* Section III, D, below, on hydroxylamine reductase). The cell-free extract of *E. coli* grown in deep standing cultures also displayed two other nitrite reductases. One was an enzyme in which DPNH served as the electron donor, and the other was a particulate system utilizing reduced benzyl viologen or $FMNH_2$ as a donor.

Pichinoty and Senez (107, 124) found that cell suspensions or extracts of *Desulfovibrio desulfuricans* reduced nitrite and hydroxylamine to ammonia with the uptake of molecular hydrogen corresponding to stoichiometric expectations (3 moles of H_2 per mole of nitrite and 1 mole of H_2 per mole of hydroxylamine). Cytochrome c_3 was implicated as a component of these enzymes. Krasna and Rittenberg (63) also observed the reduction of nitrite and hydroxylamine to ammonia by the same species with the uptake of hydrogen gas being consistent with reduction to ammonia. McNall and Atkinson (73) isolated an *E. coli* strain (Bn) which reduced nitrate or nitrite completely to ammonia at the expense of molecular hydrogen, and showed (74) that it could utilize hyponitrite, hydroxylamine, or nitrous oxide as its sole nitrogen source. The ability of certain green algae after adaptation in the dark to a hydrogen-containing atmosphere to carry out the stoichiometric reduction of nitrite to ammonia by molecular hydrogen was reported by Kessler (54).

Vanecko and Varner (145), with the use of nitrite-infiltrated wheat leaves, identified oxygen as the gas evolved upon exposure to light and concluded that photolysis of water was the primary and immediate source of reducing substance in the light reduction of nitrite, especially since $1\frac{1}{2}$ moles of oxygen were evolved per mole of nitrite reduced.

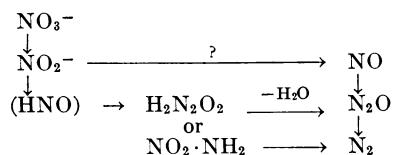$$HNO_2 + H_2O \rightarrow HN_3 + 1\frac{1}{2} O_2$$
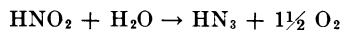
They concluded that nitrite was apparently reduced to the amino level and probably incorporated into proteins since there was no significant increase in the levels of ammonia and amide. The interesting observation by Kessler (55), with the use of intact cells of the algae *Ankistrodesmus bravnii*, that the reduction of nitrite was markedly accelerated by exposure to light under anaerobic conditions has recently been extended by Huzisige and Satoh (40a). The latter workers found that intact cells of *Euglena gracilis* experienced a doubling in the rate of nitrite reduction (aerobically or anaerobically) upon exposure to light. The addition of phosphate as well as suitable hydrogen donors such as malate or pyruvate further enhanced the reaction. Huzisige and Satoh (40b) subsequently reported the isolation from spinach leaves of a soluble enzyme (designated as photosynthetic nitrite reductase) which was required in addition to grana for the photochemical reduction of nitrite. The similarity in

the methods of preparation and the properties of photosynthetic nitrite reductase to those of photosynthetic pyridine nucleotide reductase (San Pietro and Lang (123a)) suggests that the latter enzyme is probably involved in the photosynthetic nitrite-reducing system.

B. Nitrite Reductase in Denitrifying Organisms

Denitrification is the process by which particular microorganisms convert nitrate and certain intermediate reduction products, such as nitrite, to molecular nitrogen, nitrous oxide, or nitric oxide. This process represents in part a form of nitrate respiration since the first step in the process utilizes nitrate (in place of oxygen) under anaerobic conditions for the ultimate oxidation of organic substrates. Denitrifying bacteria are facultative organisms occurring almost universally in soil and water and utilizing nitrate as a hydrogen acceptor for energy-yielding oxidative reactions. The more recent developments in this area through 1957 (including references to reviews covering details of earlier findings) were indicated by Nason and Takahashi (86). They evaluated and summarized the evidence concerning the metabolic characteristics of most denitrifyers, namely (i) that organic compounds in general serve as an energy source with either oxygen or nitrate as the ultimate electron acceptor (aromatic acids and aliphatic straight chain compounds, however, cannot be oxidized anaerobically in the presence of nitrate); (ii) that oxygen is a potent inhibitor of the denitrification process by virtue of its effective competition with nitrate as an acceptor of electrons in the oxidative functions of the cell. Pichinoty and D'Ornano (106), as stated in Section II, D on adaptation) recently pointed out, however, that the inhibitory effect of oxygen may well be due to its action in preventing both the biosynthesis and activity of the respiratory nitrate reductase; (iii) that cytochromes are involved as components in the electron transport chain of the denitrification process; and (iv) that the biochemical pathway for denitrification is believed to be essentially as follows:

$$
\begin{array}{c}
NO_3^- \\
\downarrow \\
NO_2^- \xrightarrow{\quad ? \quad} NO \\
\downarrow \qquad\qquad\qquad\qquad \downarrow \\
(HNO) \rightarrow H_2N_2O_2 \xrightarrow{-H_2O} N_2O \\
\text{or} \qquad\qquad \downarrow \\
NO_2 \cdot NH_2 \longrightarrow N_2
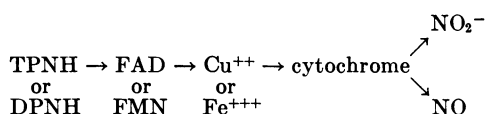\end{array}
$$

The above scheme postulates 2-electron steps. The first involves the conversion of nitrate to

nitrite, and is apparently mediated by a respiratory nitrate reductase pathway as discussed in a previous section. Fewson and Nicholas (32), on the basis of recent experiments with cell-free preparations of the denitrifying bacterium *Pseudomonas aeruginosa*, suggested a sequence of electron transport as shown previously in Section II, B2. The next step in denitrification, namely, the reduction of nitrite on the basis of a presumed 2-electron change would result in formation of the hypothetical nitroxyl intermediate, perhaps as a nitroxyl-enzyme complex. The presumed spontaneous dimerization of nitroxyl to yield possibly hyponitrite (or nitramide) might be followed by a spontaneous or enzymatic decomposition of hyponitrite involving the removal of water thereby yielding nitrous oxide (N_2O). The reduction in vitro of the latter to molecular nitrogen was demonstrated for the first time by Najjar and Chung (82) with cell-free preparations of *Pseudomonas stutzeri*. The alternate possibility that the enzymatic hydrogenation of hyponitrite (or nitramide) might lead directly to the formation of nitrogen gas seems to be more or less eliminated. The original claim by Allen and Van Niel (5) that *P. stutzeri* hydrogenated nitramide to gaseous nitrogen was subsequently ruled out by Kluyver and Verhoeven (60), who found that nitramide was decomposed too rapidly upon addition of phosphate buffer to be used as substrate. The latter workers also showed that neither *Micrococcus denitrificans* nor *Pseudomonas aeruginosa* evolved nitrogen gas from sodium hyponitrite, confirming the results of Allen and Van Niel (5) with *P. stutzeri*.

With regard to the other steps in the over-all denitrification pathway, Sacks and Barker (122) concluded that nitrous oxide was not an obligatory intermediate in the formation of molecular nitrogen since (i) nitrous oxide was not detectable in their denitrification experiments with *Pseudomonas denitrificans*; (ii) nitrous oxide utilization was selectively blocked by azide or dinitrophenol under conditions which permitted the formation of nitrogen from nitrite; and (iii) a lag frequently preceded the utilization of nitrous oxide by resting cells, a phenomenon not observed in the conversion of nitrite to N_2. Allen and Van Niel (5) also observed that the conversion in *P. stutzeri* of nitrous oxide to N_2 was inhibited by cyanide, whereas the reduction of nitrite to N_2 was unaffected.

The first report of cell-free denitrification,

namely, the enzymatic reduction of nitrate or nitrite to molecular nitrogen and nitrous oxide (as well as nitric oxide), was made by Najjar and Allen (81) with preparations from *P. stutzeri* and *Bacillus subtilis*. Chung and Najjar (20, 21, 82), with crude ammonium sulfate fractions of *P. stutzeri* extracts, subsequently showed that in the reduction of nitrite to nitric oxide TPNH and DPNH served as electron donors, and FAD and FMN gave a 2-fold stimulation. The crude enzyme fractions also contained substantial amounts of cytochrome *c* and were inhibited by several metal-bindings agents. The partial loss in activity after successive dialysis against solutions of metal-binding agents and water was restored to a small extent by the addition of cupric and iron salts. Nitric oxide reductase activity (NO₂ → N₂) in similar fractions exhibited almost identical properties. Najjar and Chung therefore suggested the following electron transport sequence to yield N₂ and NO in the reduction of NO₂⁻, and N₂ in the reduction of NO.

$$TPNH \rightarrow FAD \rightarrow Cu^{++} \rightarrow cytochrome \begin{array}{c} \nearrow NO_2^- \\ \searrow NO \end{array}$$
$$\text{or} \quad \text{or} \quad \text{or}$$
$$DPNH \quad FMN \quad Fe^{+++}$$

Walker and Nicholas (151), with the use of a 600-fold purified nitrite reductase from *Pseudomonas aeruginosa*, also reported nitric oxide to be the product of the reaction. Except for the fact that DPNH and TPNH could not be used as electron donors, the enzyme was quite similar to the corresponding *P. stutzeri* system reported previously by Najjar and Chung (82). As an FAD enzyme possessing a cytochrome *c*-type spectrum, it also contained iron and copper components. Fewson and Nicholas (34), however, questioned the claim by Chung and Najjar (21) that the nitrite oxide reductase of *P. stutzeri* required both iron and copper for activity. They believed that the action of iron, copper, and, to a smaller extent, zinc, in reactivating the enzyme after prolonged dialysis against salicylaldoxime, resulted from the removal of residual chelate bound to the enzyme, especially since the reversal of inhibition was in the order of affinities of the metals for salicylaldoxime. Fewson and Nicholas (33) also purified a nitric oxide reductase from *Pseudomonas aeruginosa* and characterized it as a flavoprotein which did not use reduced pyridine nucleotides. They claimed it contained iron but not copper, and that nitric oxide is an inter-
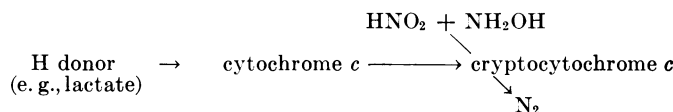
mediate not only in the nitrate respiratory pathway (e.g., denitrification), but also in the nitrate assimilatory pathway and possibly in nitrogen fixation and nitrification.

Yamanaka and Okunuki (158) isolated a highly purified cytochrome oxidase particle from *P. aeruginosa* which also catalyzed the reduction of nitrite by the reduced cytochrome *c*-like component of the bacterium. According to these results, the purified cytochrome oxidase particle of *Pseudomonas* functioned as a nitrite reductase. Iwasaki and Mori (51), working with extracts of a denitrifying bacterium tentatively identified as *Pseudononas denitrificans*, reported that in the presence of lactate, nitrite was reduced only to molecular nitrogen, whereas, in the absence of added lactate, the evolved gas was almost exclusively nitrous oxide. Hyponitrite could not be utilized but hydroxylamine had a stimulatory effect on N₂ gas evolution from nitrite. On the basis of these results they suggested that, in denitrification, nitrite is converted to N₂ by electrons which are enzymatically transferred from a substrate such as lactate. According to their hypothesis, a portion of the nitrite is reduced to a substance such as hydroxylamine which in turn reacts with the remaining nitrite in either of two presumed ways: (i) hydroxylamine undergoes an enzymatic reaction with nitrite to produce nitrous oxide; or (ii) in the presence of lactate and lactic dehydrogenase, nitrite is first reduced to NO and then reacts enzymatically with hydroxylamine to produce N₂.

$$HNO_2 \xrightarrow[\searrow H]{4H} NH_2OH \xrightarrow{+HNO_2} N_2O$$
$$\searrow NO \quad \downarrow H$$
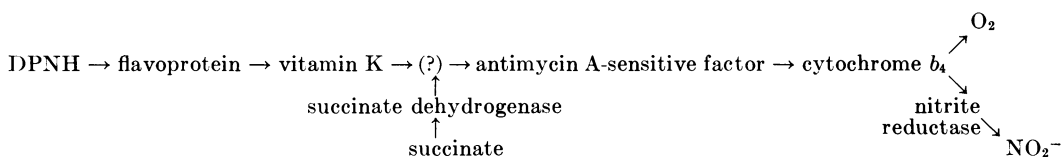$$N_2 + 2H_2O$$
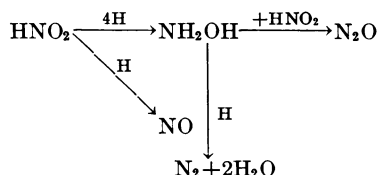
Iwasaki (50) subsequently purified two inactive fractions from the extract which upon recombination served as a denitrification system. One of the fractions had the typical absorption spectrum of a *c*-type cytochrome, and the other, which he considered to be the denitrifying enzyme itself, was red due to a so-called "cryptocytochrome *c*." The above enzymatic reaction of nitrite with hydroxylamine to produce nitrous oxide was apparently mediated by the latter fraction. The cytochrome *c*-like fraction was considered to be an electron carrier to the denitrifying or "crypto-

cytochrome c'' system with the latter also mediating the conversion of nitrite and hydroxylamine to N_2 :

and a soluble component, both of which were necessary for denitrification. The activity of the soluble component during purification paralleled

$$HNO_2 + NH_2OH$$

H donor $\quad \rightarrow \quad$ cytochrome $c \longrightarrow$ cryptocytochrome c
(e. g., lactate) $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\searrow$
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad N_2$

Another denitrifying bacterium, a halotolerant *Micrococcus* (strain 203), has also been the subject of cell-free denitrification studies. Detailed investigations by Asano of the nitrite-reducing system from this organism revealed certain differences in properties as compared to the corresponding *Pseudomonas* enzyme. Using a partially purified enzyme preparation from *Micrococcus*, Asano (6) reported that the electron transport system functioning in the reduction of nitrite was similar to the electron transport chain to oxygen. He observed that virtually all the nitrite reduced was converted to nitrogen gas with 1 mole of N_2 appearing for every 2 moles of nitrite disappearing. The preparation possessed DPNH-nitrite reductase activity in the presence of high NaCl concentrations (0.6 M), was stimulated by added FAD and menadione, and was inhibited by amytal, quinine, Dicumarol, antimycin A, and carbon monoxide. The system also appeared to include a cytochrome b_4 as an electron carrier. In view of his results, Asano suggested the following electron transport sequence for *Micrococcus* nitrite reductase.

hydroxylamine reductase activity. Using partially purified dialyzed preparations, he found (8) that the soluble and particulate components were activated by copper and iron, respectively. Asano's conclusion that neither component was of a cytochrome nature, unlike that reported for the *Pseudomonas* nitrite reductase system by Iwasaki and Mori (51), leaves unexplained his earlier proposal that cytochrome b_1 was an electron carrier as indicated in the above sequence.

C. Hyponitrite as a Possible Intermediate in Nitrite Reduction

The original reports by Medina and Nicholas (76–78) in 1957 constitute the only claim for a cell-free system which catalyzes the reduction of hyponitrite. Taniguchi et al. (141) had pointed out earlier that hyponitrous acid was not reduced by a crude extract of halotolerant bacteria containing all the enzyme systems for reduction of nitrate to ammonia with reduced methylene blue as the hydrogen donor. Medina and Nicholas indicated the presence of a DPNH-hyponitrite reductase in crude extracts of *Neurospora* and

$\qquad O_2$
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\nearrow$
DPNH $\rightarrow$ flavoprotein $\rightarrow$ vitamin K $\rightarrow$ (?) $\rightarrow$ antimycin A-sensitive factor $\rightarrow$ cytochrome b_4
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\searrow$
$\qquad\qquad\qquad\qquad\qquad$ succinate dehydrogenase $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ nitrite
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ reductase $\searrow$
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ succinate $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad NO_2^-$

The *Micrococcus* nitrite reductase preparation also possessed a powerful hydroxylamine reductase activity (in contrast to the absence of such activity in the nitrite reductase preparations of *Pseudomonas*), but nitrite was not reduced to hydroxylamine and ammonia. The observed inhibitory effect of hydroxylamine indicated that the mechanism of denitrification was different from that reported for *Pseudomonas* by Iwasaki and Mori (51) as discussed above.

Asano (7) subsequently resolved the *Micrococcus* nitrite reductase denitrifying system into two protein fractions, a particulate component

designated it as a metalloflavoprotein catalyzing the conversion of hyponitrite to ammonia. Its unusual similarity to the accompanying *Neurospora* nitrite reductase including its reported sensitivity to a number of inhibitors (e.g., naphthoquinone and 2-n-heptyl-4-hydroxyquinoline-N-oxide) as well as to a nutritional deficiency of copper or iron raises the question as to whether or not the two systems are identical.

More important, however, is the question of the stability of hyponitrite in buffered solutions near neutrality. Frear and Burrel (35) observed that although aqueous solutions of sodium hypo-

nitrite were stable for 2 hours at room temperature at pH 11.3, they spontaneously decomposed (apparently to nitrite and nitrate) at pH 7.3 at a much faster rate displaying half-lives of about 11 minutes. Chaudhary, Wilson, and Roberts (15) had already emphasized that hyponitrite was quite easily and spontaneously oxidized under neutral or slightly acidic conditions. In their own experiments on the possible reduction of added hyponitrite by infiltrated soybean leaves, Frear and Burrell (35) concluded that hyponitrite nitrogen was first partially or completely oxidized to the level of nitrite (or nitrate) prior to its biological reduction to ammonia. Their results, therefore, neither established that hyponitrite per se was assimilated nor that it was an intermediate in the reduction of nitrate or nitrite to ammonia. The previous report by Vanecko and Frear (144) that hyponitrite was an intermediate in nitrate reduction, based on the conversion of N^{15}-labeled hyponitrite into reduced nitrogen fractions by green leaves, must therefore be re-evaluated in view of the established instability of hyponitrite. The possible role, if any, of this compound in inorganic nitrogen metabolism is therefore still in a highly unsettled state.

D. Hydroxylamine Reductase

Yamagata (157) in 1939 was among the first to note hydroxylamine reductase in bacteria with the aid of an artificial dye as an electron donor. Some 15 years later, further studies of the enzyme from bacteria by Egami's group (141) and Klausmeier and Bard (58) and from Neurospora and soybean leaves (84, 159) by Nason and his colleagues were undertaken. The Neurospora hydroxylamine reductase was demonstrated to be a pyridine nucleotide-specific metalloflavoprotein which catalyzed the stoichiometric reduction of hydroxylamine to ammonia (159), whereas the corresponding pyridine nucleotide-specific metalloprotein from soybean leaves showed a specific requirement for Mn^{++} ions (84, 119). The properties of the latter system have already been discussed in Section III, A. Mn^{++} was also observed to be a highly specific activator for the flavin-stimulated adaptive pyridine nucleotide-hydroxylamine reductase of Azotobacter although the product of hydroxylamine reduction was not identified (131). Bulen (12) also independently observed a particulate pyridine

nucleotide-hydroxylamine reductase in the same organism.

Reinvestigation of "ammonium dehydrogenase" in Bacillus subtilis extracts which were reported to catalyze the reversible reduction of hydroxylamine by DPNH to ammonia, DPN, and water (Klausmeier and Bard (58)), showed that the system had only hydroxylamine reductase activity similar to that of Neurospora (Zucker and Nason, (159)). Roussos, Takahashi, and Nason (120), although confirming the observations of Klausmeier and Bard, found that the apparent enzymatic reduction of DPNH by NH_4OH resulted instead from an indirect pH effect. The results were in keeping with the calculated equilibrium constant of 10^{35} for hydroxylamine reduction by DPNH, making it unlikely that the reverse reaction could be demonstrated.
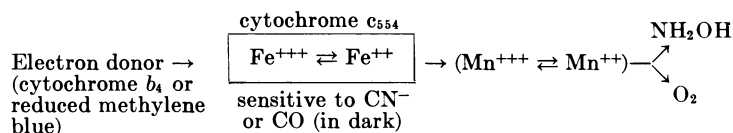
Kono, Taniguchi, and Egami (62) purified a soluble, autoxidizable, and carbon monoxide-binding cytochrome from a halotolerant bacterium. The pigment catalyzed the reduction of hydroxylamine by reduced methylene blue and was stimulated by Mn^{++}. Ishimoto, Yagi, and Shiraki (46), using cell-free extracts of sulfate-reducing bacteria, showed a cytochrome requirement for the reduction of hydroxylamine to ammonia by hydrogen. Chemically reduced cytochrome also evolved hydrogen gas in the presence of hydrogenase.

In continuing their earlier investigations (107, 126) on the reduction of hydroxylamine to ammonia by molecular hydrogen in the presence of cell-free preparations of Desulfovibrio desulfuricans, Senez and Pichinoty (124, 125) concluded that hydrogenase was the only enzymatic factor in the process. They proposed that the hydroxylamine-reducing mechanism included reduction by hydrogenase of a natural electron carrier, cytochrome c_3 (originally noted in this organism by Postgate (112, 113) as being involved in the reduction of sulfate and sulfite), which was nonenzymatically reoxidized by hydroxylamine without the intervention of a specific reductase. Although benzyl viologen could be substituted for the cytochrome, it was a less efficient electron carrier. Purified ferrocytochrome c from beef heart, like cytochrome c_3, was nonenzymatically reoxidized by hydroxylamine but its rate of oxidation was lower. On the basis of experiments with other anaerobic and aerobic bacteria, they obtained supporting evidence that the reduction

of hydroxylamine by hydrogenase and a non-specific natural or artificial electron carrier was not confined to the sulfate-reducing bacteria but could be effected by any organism possessing hydrogenase. DPN was apparently not involved since it was neither reduced enzymatically by H_2 in the presence of bacterial extracts, nor was DPNH oxidized by hydroxylamine or cytochrome c_3.

The above property of certain heme proteins to serve as electron donors or carriers in the "nonenzymatic" reduction of hydroxylamine

The 200-fold purified enzyme showed a typical cytochrome c-type spectrum, was inhibited by several metal-binding agents, and contained both iron and manganese. The inactivated, dialyzed enzyme was specifically restored by added Mn^{++} which the authors believed experienced a possible reversible change to manganic ions (Mn^{+++}). The reduced enzyme also was reported to react with O_2. On the basis of their experimental results they postulated the following electron transport sequence for the hydroxylamine reductase of *Micrococcus*:

$$\text{Electron donor} \rightarrow \begin{array}{c} \text{cytochrome } c_{554} \\ \boxed{Fe^{+++} \rightleftarrows Fe^{++}} \\ \hline \text{sensitive to } CN^- \\ \text{or CO (in dark)} \end{array} \rightarrow (Mn^{+++} \rightleftarrows Mn^{++}) \underset{O_2}{\overset{NH_2OH}{\diagup}}$$

(cytochrome b_4 or reduced methylene blue)

had already been observed by Raw (115) for mammalian cytochrome and even earlier by Colter and Quastel (22) for hemoglobin. The latter workers reported that hemoglobin under anaerobic conditions acted as an enzyme bringing about the reduction of hydroxylamine to ammonia with the concomitant oxidation of hemoglobin to methemoglobin. It also catalyzed the reduction of hydroxylamine to ammonia by cysteine or ascorbic acid. The reverse reaction was also demonstrated, namely, the oxidation of hydroxylamine to N_2 by methemoglobin to yield hemoglobin. Heat treatment of hemoglobin, or the presence of cyanide (10^{-2} M) greatly diminished its power to catalyze the breakdown of hydroxylamine. Hemin itself was far less effective than an equivalent quantity of hemoglobin in catalyzing the breakdown of hydroxylamine. The question as to whether the reaction between heme proteins and hydroxylamine is enzymatic or nonenzymatic is philosophical and not readily answered.

Kono and Taniguchi (61) purified and characterized the hydroxylamine reductase of the denitrifying halotolerant *Micrococcus* strain 203 using reduced methylene blue as the electron donor. It is of some interest that this organism and other typical denitrifiers (e.g., *Micrococcus denitrificans* and *Pseudomonas denitrificans*) possess an active hydroxylamine reductase even when strongly denitrifying.

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2 \qquad NH_2OH \rightarrow NH_3$$
$$\text{Denitrification} \qquad \text{Hydroxylamine}$$
$$\text{reductase}$$
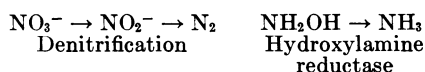
The hydroxylamine reductase of another denitrifier, *Pseudomonas denitrificans*, was purified about 50-fold by Walker and Nicholas (152) and shown to catalyze the reduction of hydroxylamine to ammonia using reduced dyes or flavins as the electron source. DPNH, TPNH, or reduced cytochrome c did not serve as electron donors. The system appeared to be an FAD, manganese enzyme possessing a cytochrome of the c type, although there was no evidence that the latter functioned in electron transfer during enzyme action. The major difference between this hydroxylamine reductase and that of *Micrococcus* appeared to be the absence of an FAD requirement by the latter.

Mager (67) has made the highly interesting observation that the TPNH-specific hydroxylamine reductase and TPNH-specific sulfite reductase activities (both are FAD dependent) of *E. coli* may well be catalyzed by the same enzyme. The facts that they followed a strictly parallel pattern of quantitative response in terms of inhibition, denaturation, feed back repression by cysteine, cystine or methionine, and stimulation of formation by serine, as well as an apparent competitive inhibition between the substrates hydroxylamine and sulfite, led him to this conclusion. The Michaelis constant for sulfite was more than 100-fold lower than the corresponding K_m for hydroxylamine, reflecting a large difference in enzyme affinity for the respective substrates. Mager is of the opinion that sulfite reduction represents the "true" physiological function, whereas reduction of hydroxylamine

"constitutes an incidental capacity of the same enzyme molecule." At most this can only be a tentative conclusion. The curious substrate specificity of the enzyme for sulfite and hydroxylamine, as well as the inhibition of catalytic function by cyanide, led to the suggestion that a free carbonyl group is the "active site" of the enzyme.

IV. REDUCTION OF ORGANO-NITRO COMPOUNDS

The metabolic significance of the reduction of organo-nitro compounds by intact cells and purified enzyme preparations is still obscure. The possibility that nitrate assimilation may proceed by way of an organic pathway, whereby an inorganic reduction product of nitrate other than ammonia is introduced into organic compounds and further reduced, was postulated by McElroy and Spencer (72). The experiments of Silver and McElroy (128), using *Neurospora* mutants blocked at different genetically controlled steps in nitrite utilization, suggested a pathway leading from hydroxylamine through pyridoxaloxime phosphate and pyridoxamine phosphate to amino nitrogen. The mutants required pyridoxine in the media for growth on nitrite but not on ammonia. They proposed that an alternative inorganic assimilative pathway via hydroxylamine and ammonia supplemented the organic route.

Nicholas et al. (89) subsequently reported that the pyridine nucleotide-nitrite reductase was activated in vitro in *crude* extracts of the pyridoxine-requiring mutant of *Neurospora* by adding either pyridoxine, pyridoxal, or pyridoxal phosphate. Although they reported that this effect in vitro was not obtained with the *purified* enzyme, they did not indicate whether the purified enzyme was prepared from the wild-type strain or the mutant. In contrast to the mutant which required pyridoxine only when grown on nitrite or nitrate (but not on ammonia) used by Silver and McElroy, the mutant employed by Nicholas *et al.* also displayed a vitamin B_6 requirement when grown on ammonia. The latter workers, in addition, indicated that they found no evidence for the enzymatic reduction of oximes to amino acids by *Neurospora*.

McElroy and Spencer (72) also considered that the pyridine nucleotide-nitroaryl-reducing system might be utilized in the organic pathway. The characteristics of a number of enzymes such as xanthine oxidase, diaphorase, L-amino oxidase, hydrogenase, cytochrome *c* reductase,

several unidentified pyridine nucleotide-linked flavoproteins and a number of molybdenum-dependent enzymes in catalyzing the reduction of various nitroaryl compounds (including picric acid, nitrofurazone, and chloramphenicol), nitroprusside, and methemoglobin were discussed in earlier reviews (83, 86). The possibility was presented that these compounds, like methylene blue and indophenol, were simply nonspecific electron acceptors for numerous enzymes, especially for flavoprotein systems. Virtually no new work has been reported in this area since 1957. Cain (13) recently isolated species of *Nocardia* and *Pseudomonas* from soil and polluted streams which were capable of metabolizing nitrobenzoic acids. *o*- and *p*-Nitrobenzoate under aerobic conditions could provide the sole source of nitrogen as well as carbon for two *Nocardia* species, resulting in a rapid initial ammonia production. The process of oxidation of both *o*- and *p*-nitrobenzoic acids was an adaptive one and was competitively inhibited by the *m*-isomer. Gunderson and Jensen (36) had previously obtained a strain of *Corynebacterium simplex* from the soil which could utilize the herbicide 4,6-dinitro-*o*-cresol as its sole source of carbon and nitrogen. More recently, Villanueva (147) prepared a 200-fold purified enzyme from a *Nocardia* species which catalyzed the reduction of *p*-dinitrobenzene by DPNH.

V. CELL-FREE NITRIFICATION

Important breakthroughs have taken place in our heretofore meager knowledge of the biochemical pathways and mechanisms of autotrophic nitrification since this area was last summarized and evaluated 3 years ago by Nason and Takahashi (86). The primary reason for this unusual progress originated from the success of Aleem and Alexander (1) in growing adequate quantities of nitrifying organisms in pure culture. Thus it made available sufficient experimental material for cell-free studies.

Aleem and Alexander (1) demonstrated for the first time a nitrite-oxidizing system in cell-free *Nitrobacter* extracts and showed that it was stimulated by the addition of iron and inhibited by low concentrations of cyanide. Aleem and Nason (2) subsequently found that the nitrite-oxidizing activity in cell-free preparations of *Nitrobacter* resided solely in a cytochrome-containing particle designated as nitrite oxidase. Their data implicated the action of the nitrite

oxidase system to involve the enzymatic transfer of electrons from nitrite to molecular oxygen via cytochrome c- and cytochrome oxidase-like components according to the following sequence:

$$NO_2^- \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a_1 \rightarrow O_2$$
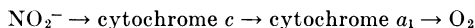
Although a specific requirement for iron could be consistently demonstrated by means of the cyanide-dialysis procedure of Nicholas and Nason (90) the role of iron and its site of action in the above electron transport chain has not yet been established. Thus far it has not been possible to observe a flavin component spectrophotometrically or to show a flavin requirement for the nitrite oxidase system. Aleem and Nason (3) also showed that partially purified nitrite oxidase particles catalyzed the formation of high energy phosphate bonds as ATP (when ADP was used as the phosphate acceptor) concomitant with the specific enzymatic oxidation of nitrite by molecular oxygen. Inosine diphosphate and guanosine diphosphate served as phosphate acceptors in place of ADP, whereas uridine diphosphate and cytidine diphosphate were ineffective. The highest P:O ratios attained thus far with nitrite as the oxidizable substrate were about 0.2. It is quite possible that this value may be substantially increased as the system is further characterized and more favorable conditions are found. The electron transport system mediating nitrite oxidation is sensitive to relatively high concentrations of such respiratory-chain inhibitors as antimycin A and 2-n-heptyl-4-hydroxyquinoline-N-oxide. Dinitrophenol, thyroxine, and Dicumarol, however, failed to uncouple the nitrite-specific oxidative phosphorylation. Malovolta, Delwiche, and Burge (69) also observed phosphate esterification accompanying nitrite oxidation with crude cell-free extracts of *Nitrobacter* and indicated that the incorporation of C^{14}-labeled CO_2 into organic components resembled that of photosynthesis. More recently Aleem and Nason (*unpublished data*) partially purified and characterized a nitrite-cytochrome c reductase from *Nitrobacter* which was inhibited by metal-binding agents and was apparently different from pyridine nucleotide-cytochrome c reductase. The further transport of electrons from cytochrome c to O_2 was catalyzed by the cytochrome oxidase present in the extracts.

Imshenetskii, Ruban, and their colleagues reported in a series of papers some 5 years ago (43–45) their observations of cell-free oxidation of ammonia and hydroxylamine to nitrite by autolysates and filtrates of *Nitrosomonas europea*. Unfortunately, no precautions were indicated to eliminate unbroken cells or to avoid contamination by other microorganisms, which was especially likely in view of incubation periods ranging as long as 5 days at 37 C.

Nicholas and Jones (88) recently found that cell-free extracts of the same species catalyzed the oxidation of hydroxylamine but not of ammonia to nitrite, provided a suitable acceptor such as cytochrome c or phenazine methosulfate was added. When cytochrome c was the acceptor, phosphate was required, but at higher concentrations it proved to be a competitive inhibitor with cytochrome c. Hydrazine when used in place of hydroxylamine enzymatically reduced cytochrome c but no nitrite was produced. The enzyme was purified about 40-fold. Delwiche, Burge, and Malovolta (25) recently indicated that phosphate esterification accompanied hydroxylamine oxidation by cell-free extracts of *Nitrosomonas*.

The recent observations by Creswell and Hewitt (23), with a partially purified enzyme from extracts of marrow plants which catalyzed the oxidation of hydroxylamine, can probably best be ascribed to the Mn-dependent peroxidase systems elucidated by Kenten and Mann (52, 53). O_2 or H_2O_2 was required and horseradish peroxidase was found to replace the enzymatic component of the marrow leaf preparation, when boiled leaf extract and manganese were also present. The system is analogous to that previously demonstrated by Heppel and Porterfield (37a) in which nitrite was oxidized to nitrate by the peroxidase-like action of liver catalase.

The area of nitrification has recently been reviewed by Aleem and Nason (4). The important question as to how *Nitrosomonas* and *Nitrobacter* cells obtain their reducing power, which is presumably derived from ammonia and nitrite oxidation, respectively, is still untouched. If we assume that electrons provided by ammonia and nitrite ultimately reach the level of pyridine nucleotides or succinate, then energy must be provided to make possible the transfer of their electrons "uphill" in view of the high positive potential of these substrates (viz., ammonia and nitrite).
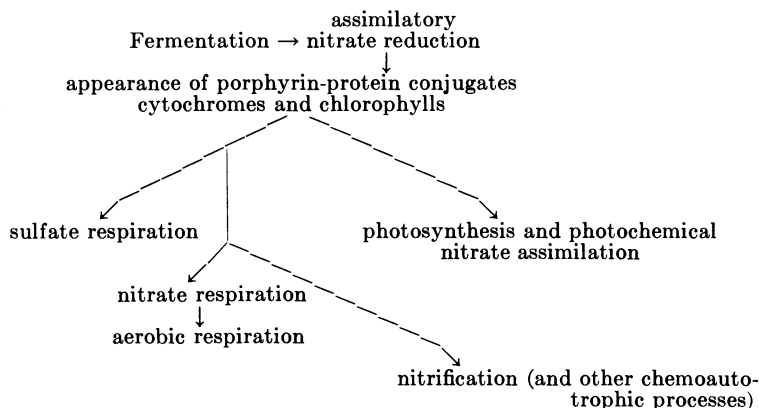
VI. Probable Evolution of Inorganic Nitrogen Pathways

Several proposals have been recently made with regard to the evolutionary development of the various pathways of inorganic nitrogen metabolism and their significance in the economy of the organism (47, 129). At our present stage of knowledge they represent at best rational speculations based for the most part on current biochemical information. A number of important questions, however, remain unanswered.

According to the reasonable and well constructed hypothesis of the origin and evolution of life presented by Oparin (103), anaerobic heterotrophic organisms were the forerunners of the aerobic forms of life because the latter were dependent upon the accumulation of free oxygen in the atmosphere at the expense of photosynthesis. If one further assumes that the appearance and functioning of porphyrin-conjugated proteins (e.g., cytochromes and chlorophylls) occurred not in the earlier periods of heterotroph development but just prior, relatively, to the development of the first photosynthetic organisms, then the following sequence in the evolution of nitrate reduction seems reasonable.

oxidizing DPNH. The subsequent evolution of light-sensitive pigmented organisms capable of imparting a high reduction potential to the hydrogen of water (and possibly to other substances) to produce reduced pyridine nucleotides presumably accounts for the phenomenon of photochemical nitrate assimilation. The evolution of cytochromes, presumably starting with a low potential cytochrome as exemplified by the cytochrome c_3 of *Desulfovibrio desulfuricans* (112, 113), was probably succeeded by the development of cytochromes of higher and higher redox potential to yield nitrate respiratory electron chains. This in turn could have easily evolved into an aerobic respiratory pathway in which molecular oxygen replaced nitrate. The chemoautotrophs by virtue of their absolute requirement for free oxygen (with the exception of *Thiobacillus. denitrificans*) and their allegedly highly advanced morphological characteristics are presumed to have arisen later than the photoautotrophs or photosynthetic forms (129).

One of the major discrepancies of the above evolutionary scheme has to do with the origin of nitrate. If it is assumed that prior to the origin and evolution of photosynthesis, nitrogen, like


```
                               assimilatory
              Fermentation → nitrate reduction
                                    ↓
            appearance of porphyrin-protein conjugates
                  cytochromes and chlorophylls


     sulfate respiration                  photosynthesis and photochemical
                                                nitrate assimilation

              nitrate respiration
                     ↓
              aerobic respiration

                                      nitrification (and other chemoauto-
                                              trophic processes)
```

In view of its lack of a cytochrome component as well as its independence of molecular oxygen, the nitrate reduction pathway of the assimilatory type seems to be quite primitive. In addition to providing a means of reducing nitrate to the ammonia level for the ultimate synthesis of proteins, it can be speculated that by virtue of being a pyridine nucleotide-linked system, it may well have served in facilitating fermentation by

many of the elements constituting the earth's environment, was in a reduced state as represented by ammonia, we are unable to account satisfactorily for the presence of large quantities of nitrate without invoking molecular oxygen. How did nitrate arise in a reducing atmosphere unless of course it was derived after the evolution of photosynthesis? Did nitrate respiration therefore precede aerobic respiration? How do the

actual biological energetics in terms of ATP formation compare between these two processes? These are some of the questions that are in need of an answer.

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